The flow cytometry approach to the analysis of biological nanoparticles

(or how to squeeze a flow cytometer like an orange)

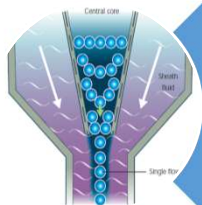
Valentina Tirelli and Massimo Sanchez

Nano Innovation 2020

Rome, 17 september 2020

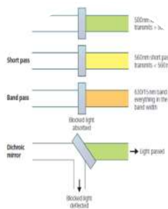
How the flow cytometer works

Cell populations and particles are analyzed or purified on the basis of their fluorescent signals or light scattering characteristics.



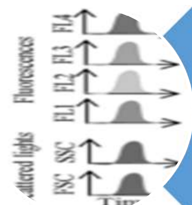
Fluidic system:

particles in solution pass one by one through the stream….



Optic system:

…Flow cytometer exploits scattered and fluorescent light signals emitted by cells excited by lasers as light sources

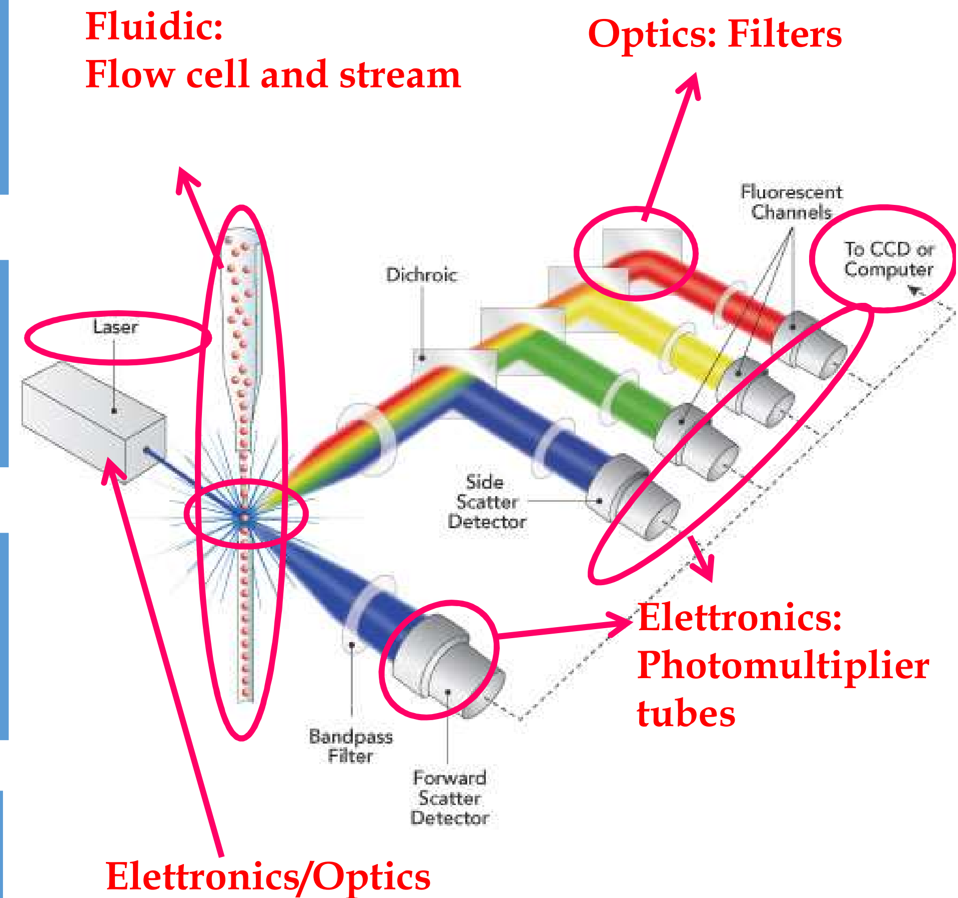


Electronic system

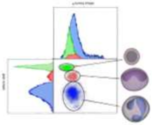
……photodiode and photomultiplier amplify and convert light signals (photons) to electric signals…

Data analysis

this pulse is digitalized and recorded to be analysed with a dedicated software



Flow Cytometry: a multidisciplinary technique



Biomedical Field

- Volume and morphological complexity of cells
- Cell surface antigens (CD markers)
- DNA (cell cycle analysis, cell kinetics, proliferation etc.)
- RNA
- Chromosome analysis and sorting
- Intracellular antigens (cytokines, secondary mediators etc.)
- Nuclear antigens

- Enzymatic activity
- pH, intracellular ionized calcium, magnesium, membrane potential
- Membrane fluidity
- Apoptosis (measurement of DNA degradation, mitochondrial membrane potential, permeability changes, caspase activity)
- cell viability
- monitoring electropermeabilization of cells
- oxidative burst
- characterising multi-drug resistance (MDR) in cancer cells
- glutathione

- **Industrial processes (milk, wine** production,,…)
- **Environment (microbiology)**
- **Public health (tap water)**

- **Agriculture and food**
- **Veterinary applications**

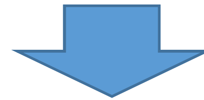
NON CONVENTIONAL Flow Cytometry

Analysis and sorting of nanoparticles

2020

Why Flow Cytometry?

There is a need for the analysis of nanoparticles



- Flow cytometry (FCM) is becoming a key technology in the study and characterization of micro-nano sized particles and extravesicles.

Why Flow Cytometry?

- Acquisition and analysis of a high number of events (>10^6 events/sample)
- Analysis of rare events
- Reduced acquisition times (>10^6 events/min)
- Choice of the population to be acquired and analyzed (gating strategies)
- Quantitative results
- Simultaneous acquisition of several parameters per events
- Purification of selected population

Analysis of nanovescicles

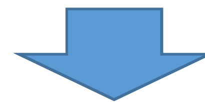
PITFALLS

Nanovescicles are below the detection limit of conventional instruments

The currently available commercial instruments are not designed to discriminate particles at sub-micron scale

Conventional FC: threshold on size (FSC)

It is possible to work around this problem with strategies involving the use of appropriate discriminator (threshold) and gating strategies to characterize nano-extracellular vesicles

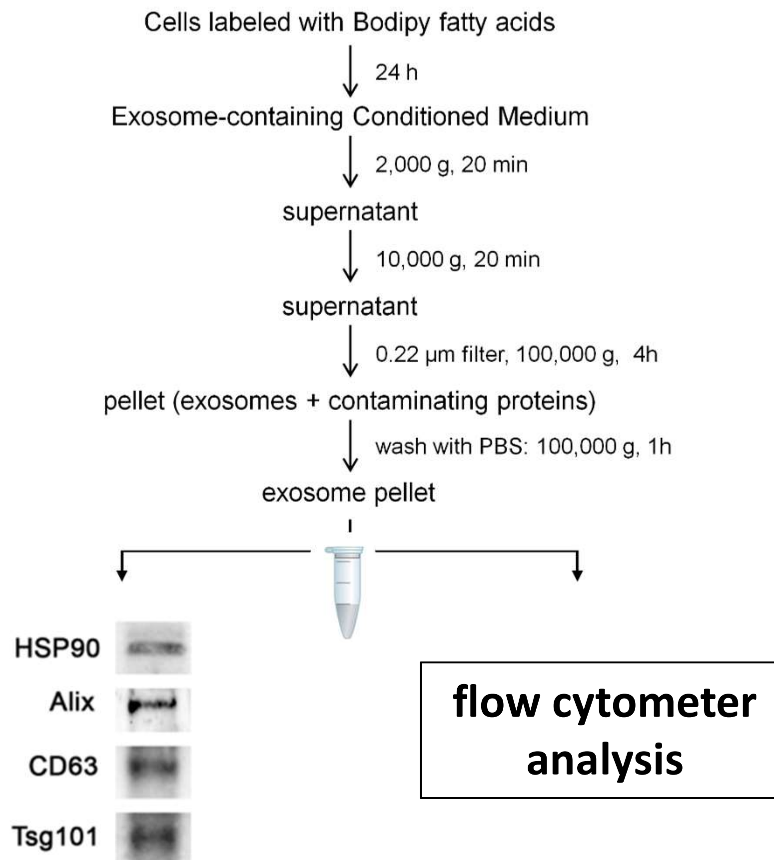


NON Conventional FC: Other strategies

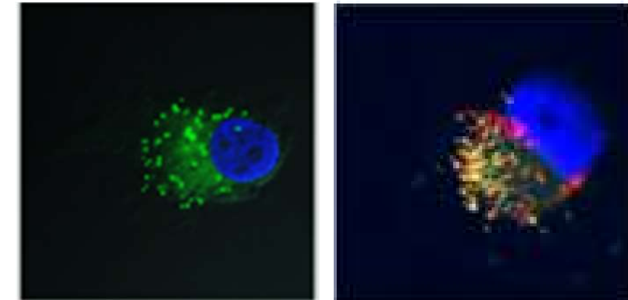
Generation, Quantification, and Tracing of Metabolically Labeled Fluorescent Exosomes.
Coscia C, Parolini I, Sanchez M et al. Methods Mol Biol. 2016;1448:217-35. doi: 10.1007/978-1-4939-3753-0_16.

Analysis of BODIPY FL C16-exosome.

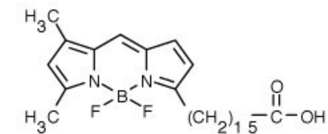
Me501 cell line



WB of exosome markers



Colocalization of C16 with anti-bis(monoacylglycero)phosphate (BMP) antibody, an exosome-specific lipid



BODIPY™ FL C16 (4,4-Difluoro-5,7-Dimethyl-4-Bora-3a,4a-Diaza-s-Indacene-3-Hexadecanoic Acid)

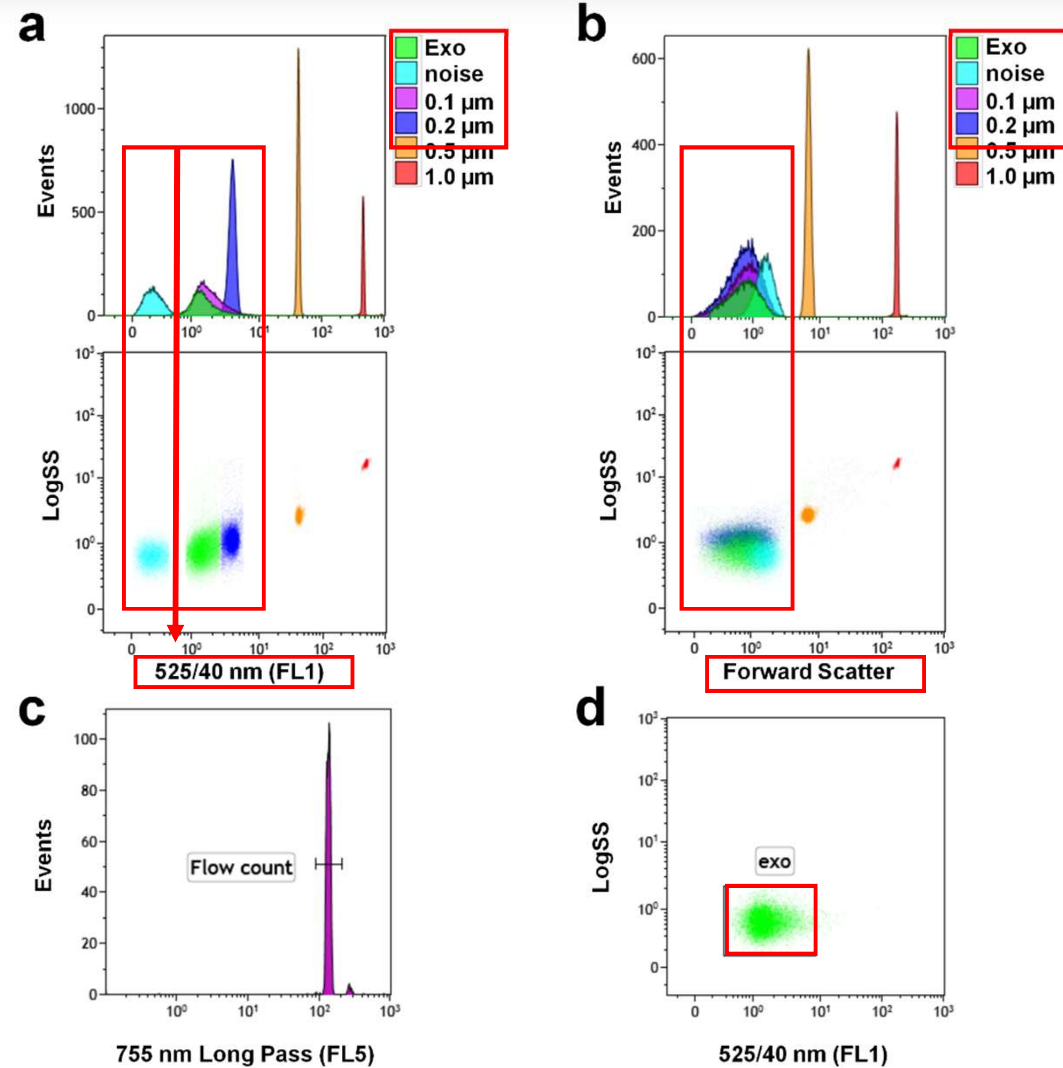
Analysis of BODIPY FL C16-exosome.

Defining the threshold

Fluorescent beads ranging from 0.1 to 1.0 µm size and background noise were analyzed for fluorescence (a, 525/40 nm FL1) and size (b)

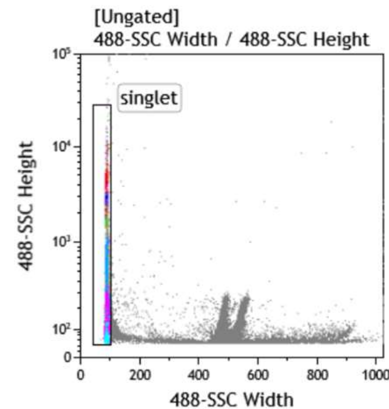
The best channel to set the threshold is that wich allows the best separation: in this case the FL1 channel.

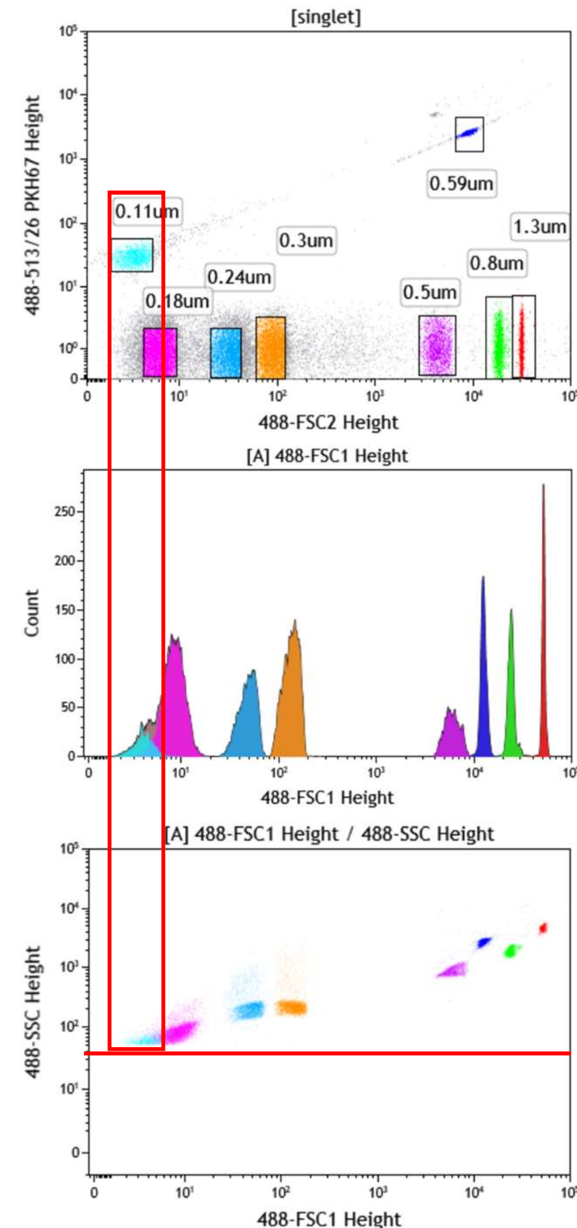
We note that the Evs scatter overlaps the electronic noise signal.



FLUORESCENCE BECOMES ESSENTIAL FOR IDENTIFICATION OF NANOPARTICLES

Strategy for sorting (I)





- **Threshold on SSC**

  Lower background than FSC but non linearity in particle size

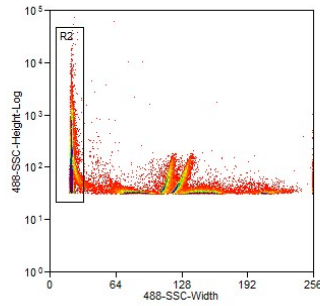
  Calibration beads to define the appropriate side scatter (SSC) threshold. The SSC threshold was applied to include particles < 100 nm.

- **Gating strategy**

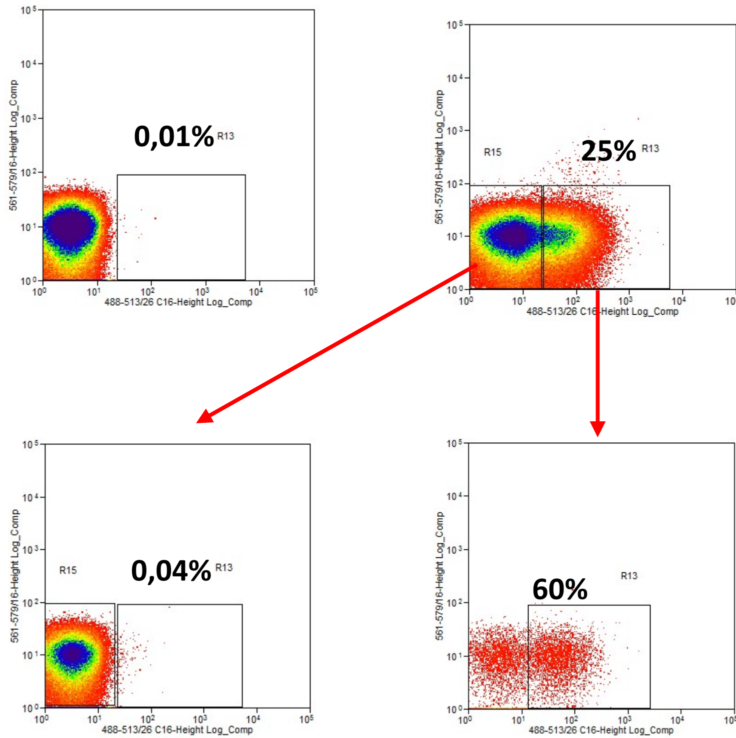
  to reduce vesicle aggregates and background noise from the sorted populations.

- **Fluorescence**

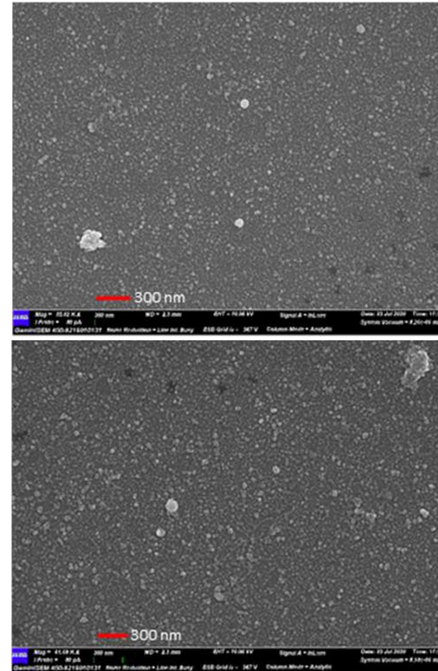
  To enhance specificity and to select the particles of interest



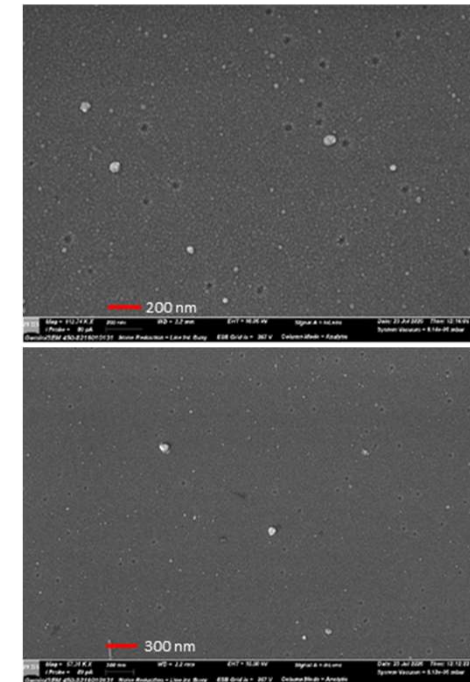
Unstained exosomes **BODIPY FL C16-exosomes**



Before sorting FE-SEM



After sorting FE-SEM



At ultrastructural level, is evident a strong background which does not allow to highlight the smaller exosomal vesicles

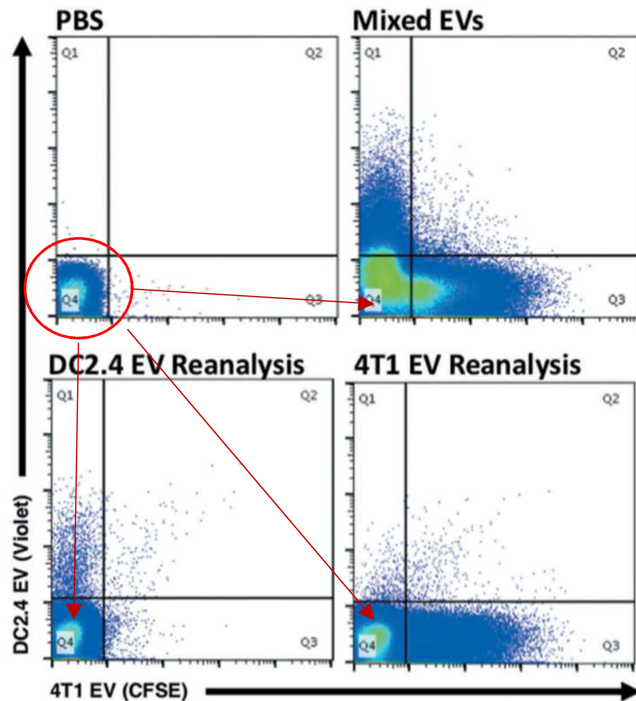
The images show the high-quality selection of the C16-bodipy positive exosomes

GEMINISEM450 - ZEISS (field emission scanning electron microscopy).

High-fidelity detection and sorting of nanoscale vesicles in viral diseases and cancer

Aizea Morales-Kastresana, Thomas A. Musich, Joshua A. Welsh, William Telford, Thorsten Demberg, James C. S. Wood, Marty Bigos, Carley D. Ross, Aliaksander Kachynski, Alan Dean, Edward J. Felton, Jonathan Van Dyke, John Tigges, Vasilis Toxavidis, David R. Parks, W. Roy Overton, Aparna H. Kesarwala, Gordon J. Freeman, Ariel Rosner, Stephen P. Perfetto, Lise Pasquet, Masaki Terabe, Katherine McKinnon, Veena Kapoor, Jane B. Trepel, Anu Puri, Hisataka Kobayashi, Bryant Yung, Xiaoyuan Chen, Peter Guion, Peter Choyke, Susan J. Knox, Ionita Ghiran, Marjorie Robert-Guroff, Jay A. Berzofsky & Jennifer C. Jones.

Journal of Extracellular Vesicles, 8:1, 1597603, DOI: 10.1080/20013078.2019.1597603

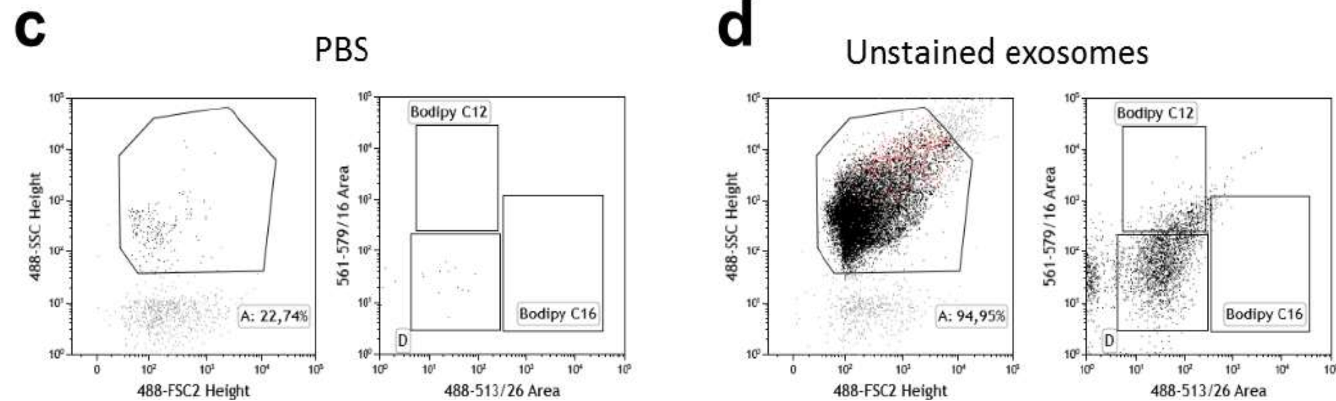


High background noise

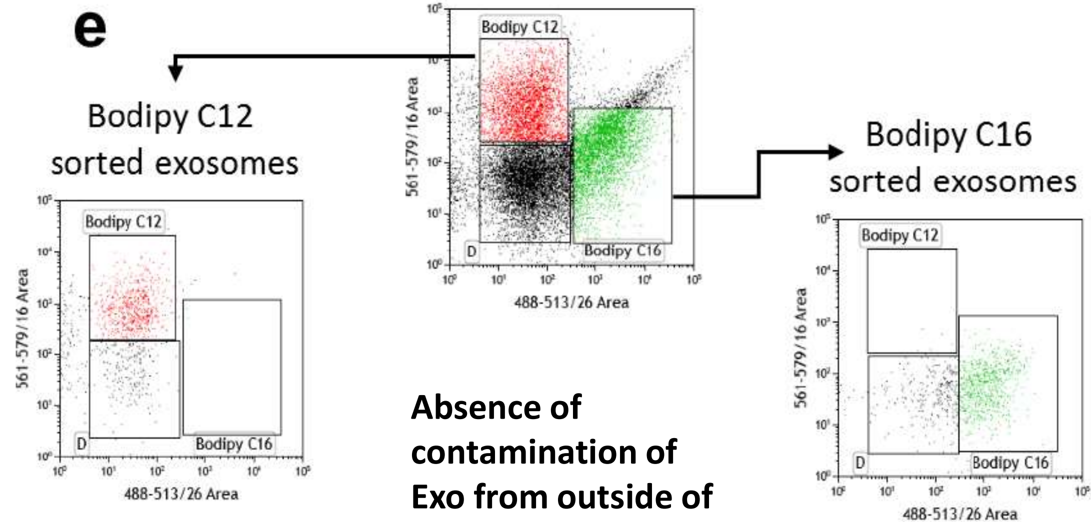
	# Events			
	Q1	Q2	Q3	% Purity
PBS	14	0	95	NA
Mixed	51189	1179	188044	NA
4T1 EV Reanalysis	2438	150	545	79
DC2.4 EV Reanalysis	433	316	65955	99

Strategy for sorting (II)

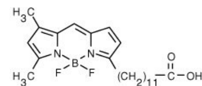
Analysis and sorting of nanovesicles produced by human melanoma cell line



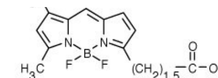
Mix of Bodipy C12/C16-Exo



Absence of contamination of Exo from outside of sorted gate



BODIPY™ FL C12 (4,4-Difluoro-5,7-Dimethyl-4-Bora-3a,4a-Diaza-s-Indacene-3-Dodecanoic Acid)



BODIPY™ FL C16 (4,4-Difluoro-5,7-Dimethyl-4-Bora-3a,4a-Diaza-s-Indacene-3-Hexadecanoic Acid)

Strategy for sorting (II)

C16 SORTED EXOSOME UPTAKE

293 CELLS + C16 sorted exosomes (3h uptake)

DAPI | **C16** | **merge** | **Transmission** | **merge + transmission**

Central optical section

Bar, 10 micron

LSM 980 - ZEISS (confocal laser scanning microscopy).

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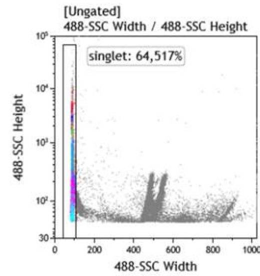
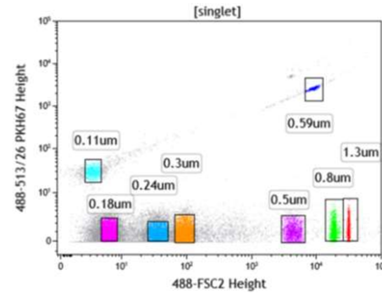
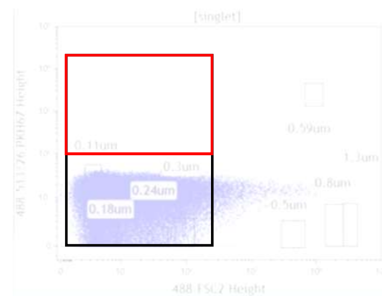
Strategy for sorting (II)

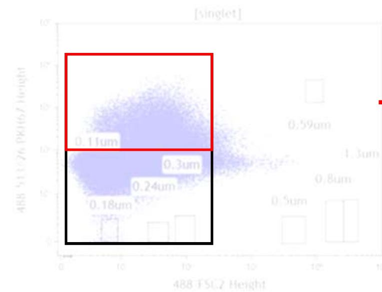
IT COULD WORK!!

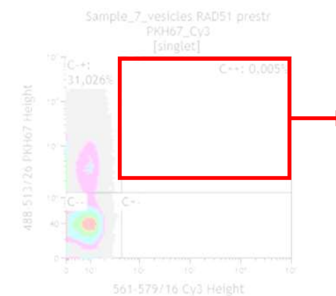
Strategy for sorting (III)

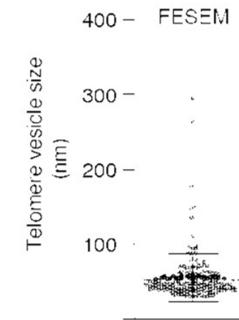




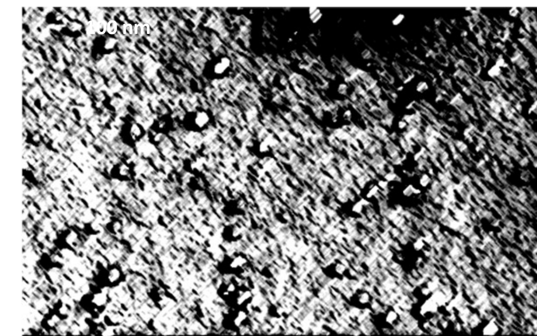








field emission scanning electron microscopy (FESEM)



magnification 100,000X

Functional studies demonstrate that we separated intact Evs containing telomers

Importance of a sorting method to preserve the integrity of Evs
(howing to the information they carry)

Conclusions

The analysis of nanovesicles by flow cytometry is not easy
but it is possible to design the right strategy

If you are able to see a stable fluorescent signal with dilutions resulting in a lower event count, you can gain more certainty over the assumption that you're analyzing (mostly) single events.

Flow Cytometry is still in its development stages, but techniques and instrumentation are rapidly improving to make this application more accessible in the near future.

No technology is currently available that gives a complete characterization of nanoparticles and reliable results

Harmonization and cooperation between different technological approaches and strategies are necessary.

Conventional FC of Individual EVs: Pitfalls

- Failure to report instrument and assay details
- Lack of instrument sensitivity, assay specificity
- Failure to calibrate fluorescence
- Failure to run key controls
- Irreproducibility, artifacts!!!

Cytometry

Fluorescent Particles in the Antibody Solution Result in False TF- and CD14-Positive Microparticles in Flow Cytometric Analysis

Hans Christian D. Aass,* Reidun Øvstebø, Anne-Marie S. Trøseid, Peter Kierulf, Jens Petter Berg, Carola Elisabeth Henriksson

Cytometry

Calcium-Phosphate Microprecipitates Mimic Microparticles When Examined with Flow Cytometry

Michael C. Larson,[1,2] * Maia R. Luthi,[3] Neil Hogg,[1] Cheryl A. Hillery[2,4]

SHORT COMMUNICATION

High-speed centrifugation induces aggregation of extracellular vesicles

Romain Linares[1], Sisareuth Tan[1], Céline Gounou[1], Nicolas Arraud[1] and Alain R. Brisson[1,2*]

[1]Molecular Imaging and NanoBioTechnology, University of Bordeaux, Pessac, France; [2]Institut Universitaire de France, Paris, France

Cytometry

A Trigger Channel Threshold Artifact in Nanoparticle Analysis

John P. Nolan,* Samuel A. Stoner

Source: https://www.beckman.it/resources/research-areas/nanoscale/measuring-single-evs

Measuring Extracellular Vesicles by Conventional Flow Cytometry: Dream or Reality?

Lucchetti D, Battaglia A, Ricciardi-Tenore C et al. Int J Mol Sci. 2020 Aug 29;21(17):E6257. doi: 10.3390/ijms21176257.

OUR THANKS GO TO:

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✉ Cintia Carella

✉ Luca Pasquini

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https://corefacilities.iss.it/dw/doku.php